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NOTES

The c-*fos* Cyclic AMP-Responsive Element Conveys Constitutive Expression to a Tissue-Specific Promoter

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The c-*fos* and cardiac α-actin promoters share homologous 5′ protein binding elements that are essential for serum-inducible and tissue-specific expression, respectively. Additional elements, auxiliary proteins or factor modifications, must distinguish the individual transcriptional responses of these two promoters. An element in the c-*fos* basal promoter that is normally responsible for transient stimulation of the *fos* gene in response to Ca^{2+} or cyclic AMP (CRE) may be able to modulate the expression of the upstream elements. We report here that this element, when inserted into the cardiac α-actin promoter, conveys constitutive expression to this otherwise highly restricted promoter. Additional data support the proposal that the CRE binding protein creates an alternative pathway whereby upstream regulatory elements in the cardiac α-actin promoter can activate transcription in a manner which circumvents the requirement for a tissue-specific environment.

The expression of a gene in an inducible or cell type-specific manner must at some stage involve either the production of unique or uniquely modified regulatory proteins by the cell or a change in the abundance of ubiquitous regulatory protein(s). Such a protein(s) then functions by interacting with other, constitutive proteins to form an activated transcription complex in a manner that is directed by the unique organization of regulatory DNA elements within the gene. In some instances such proteins and DNA elements have been identified (12, 13, 15), and an overall, yet complex, picture for transcriptional regulation is emerging.

We previously described analyses of the promoter of the human cardiac α-actin (HCA) gene (10, 11, 16). This promoter is active exclusively in cells that are committed to the myogenic lineage and contains multiple protein binding elements upstream of the TATA box. Sequences downstream of −117 from the transcription start site are sufficient to convey muscle-specific expression onto a heterologous reporter gene (16). This expression requires the presence of a decameric sequence element of consensus $CC(A/T)_6GG$, designated a CArG box, that is 100 base pairs (bp) upstream of the transcription start site. Both cardiac and skeletal α-actin promoters contain multiple, highly conserved copies of these elements (16, 19, 25, 27), and our original promoter deletion analyses suggested that the control of tissue-specific expression may reside in the CArG elements (16). Gel retardation and footprint analyses, however, have demonstrated that the proteins which bind to the HCA promoter appear to be constitutive and present in all cell types (10, 11). In particular, the CArG binding protein (CBF) of the essential −100 HCA CArG box currently appears to be indistinguishable from the non-tissue-specific c-*fos* serum response factor (2). An additional auxiliary factor appears to interact with the c-*fos* serum response factor complex (22); it is not currently known what, if any, additional factors interact with the CArG binding protein. If serum response factor and CBF1 are in fact identical, then flanking sequences, secondary protein interactions, or additional upstream or downstream motifs must modulate the transcriptional activation in the HCA and c-*fos* promoters. The differences cannot be purely a result of spatial arrangements (c-*fos* serum response element and HCA CArG1 are 300 and 100 bp, respectively, upstream of their transcription start sites), since insertion of 100 to 280 bp of pBR322 sequence between CArG1 and TATA in the HCA promoter merely serves to reduce transcription (17) without changing tissue specificity (data not shown).

In this report we show that insertion of a 26-bp element, including the 8-bp cyclic AMP-responsive element (CRE) and flanking sequence, from the c-*fos* basal promoter (23, 30) at two different sites of the HCA promoter completely eleminated the tissue-specific restriction for expression directed by the promoter. Deletion analyses demonstrated that upstream regulatory elements contributed the same degree of transcriptional activation in the modified promoter as in the wild-type promoter, but in a non-tissue-specific manner. In the absence of upstream sequences or cyclic AMP induction (3), the CRE element did not confer significant activity to HCA basal expression. This suggests that upstream protein binding elements in the HCA promoter, including the CArG boxes, are not in fact muscle specific and that the c-*fos* CRE element may be the binding site for an activator or communicator factor for upstream CArG-like elements.

Oligonucleotide 26-mers homologous to the −60 basal element of the murine c-*fos* promoter were inserted into the HCA promoter at the *Tth*IIIi site (Fig. 1). The c-*fos* element contains two sequences (underlined in Fig. 1) homologous to putative Sp1 (14) and CRE (5, 18, 23) protein binding sites. Mutations were introduced into either CRE or Sp1 sites to produce oligonucleotide *f*2 or *f*3, respectively. HCA promoter-chloramphenicol acetyltransferase (CAT) constructs con-

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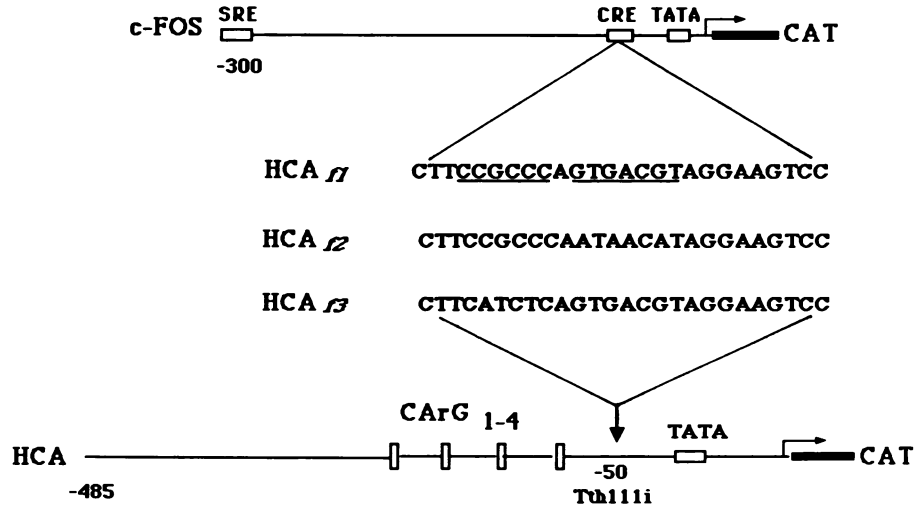

FIG. 1. Insertion of wild-type and mutated c-*fos* elements into the HCA promoter. Oligonucleotide 26-mers homologous to the murine c-*fos* basal element (8) were synthesized on a DNA synthesizer (Applied Biosystems). After annealing, the double-stranded oligonucleotides were cloned into the HCA promoter at the *Tth*111i site, which is 50-bp upstream of the transcription start site. This positions the CRE in relation to the TATA box exactly as it is positioned in the c-*fos* promoter. SRE, Serum response element.

taining the wild-type and mutated c-*fos* elements are designated HCA_{f1}, HCA_{f2}, and HCA_{f3} (Fig. 1).

Figure 2 shows transient expression of the CAT gene directed by each of the constructs in myogenic mouse C2 cells (29) and human HeLa cells. As expected, in the C2 cells all the constructs expressed at high levels, comparable to the

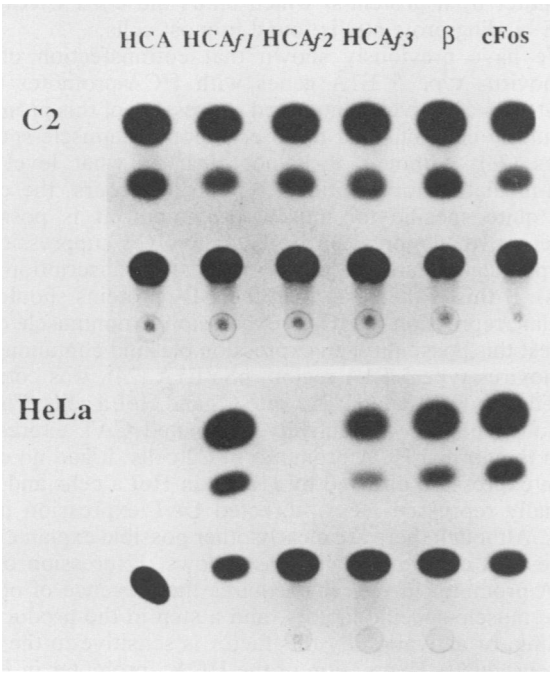

FIG. 2. Expression of HCA_{f1} in C2 and HeLa cells. Transient expression from calcium phosphate-transfected constructs was measured as previously described (16) by using 5 μg of reporter construct and 5 μg of carrier pUC18 DNA. Cells were transfected on 100-mm-diameter plates in Dulbecco modified Eagle medium plus 20% fetal calf serum and harvested at 48 h after transfection. Extracts were prepared by freeze-thaw disruption of cell pellets, and standard assay of CAT activity using [^{14}C]chloramphenicol was as described previously (16). Equal amounts of protein were assayed in each reaction. β, β-actin–CAT; cFos, c-fos_{2000}–CAT.

expression of β-actin–CAT (9) and c-fos_{2000}–CAT (4). In contrast, in HeLa cells the wild-type HCA promoter did not express above basal level, but HCA_{f1} and HCA_{f3} expressed at high levels that were comparable to β-actin–CAT and c-*fos*–CAT expression. On the other hand, HCA_{f2}, which has mutations in the CRE element, did not express above basal level in HeLa cells. Similar results were seen in mouse fibroblast NIH 3T3 cells. Thus, tissue specificity of the HCA promoter was eliminated by insertion of c-*fos* wild-type and *f*3 oligonucleotides containing intact CREs but not by insertion of the *f*2 oligonucleotide. These results represent a deviation from the classical experiments in which an upstream element confers tissue-specific expression onto a basal promoter. It is somewhat unusual for a basal element to dictate the character of the upstream regulatory elements.

To determine whether these effects correlate with protein binding to the c-*fos* elements, gel mobility shift assays (1, 7) were carried out (Fig. 3). Two apparently specific shifted bands were obtained with *f*1 and *f*3 oligonucleotides (Fig. 3, arrows). The strong lower band and faint upper band were not detected when the *f*2 probe was used, and excess unlabeled *f*2 probe did not compete with the *f*1 and *f*3 shifts. These shifted bands presumably reflect protein binding to the CRE. Protein binding to the c-*fos* basal element has been reported previously (8). An additional strong band with mobility intermediate between the two *f*1 and *f*3 probe bands was present when the *f*2 probe was used. There was no competition for this band by *f*1 or *f*3 oligonucleotides, and it probably reflects a new protein binding site created by the *f*2 base changes.

The elimination of tissue-specific expression of the HCA promoter by the c-*fos* element therefore correlates with an intact CRE and with factor binding. Since the modified HCA promoter now directs equally high levels of expression in myogenic and nonmyogenic cells, it is appropriate to ask whether upstream elements in the modified promoter still regulate the non-tissue-specific expression. To do this, 5′ deletions were made in the HCA_f promoter, and transient CAT expression was determined in C2 and HeLa cells (Fig. 4). The biphasic activity profiles of CAT expression directed by the HCA promoter in C2 cells and by the HCA_{f1} promoter

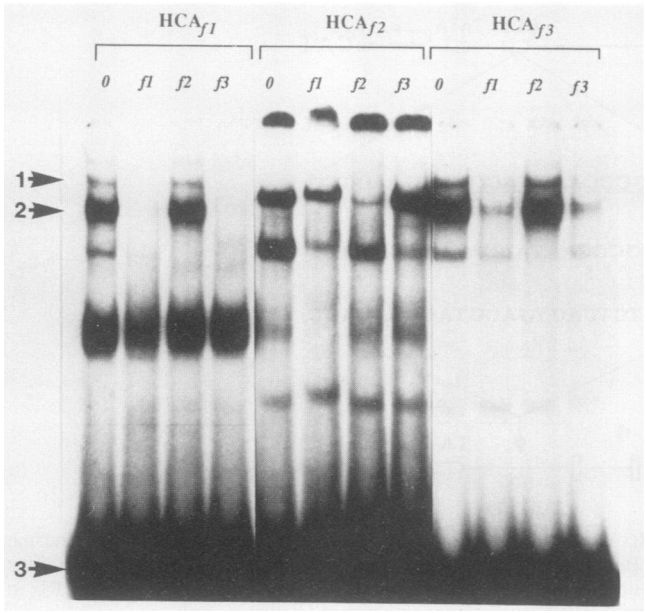

FIG. 3. Specific protein binding to the c-*fos* oligonucleotides. Oligonucleotide probes were as described in the legend to Fig. 1. Nuclear extracts were prepared from C2 myoblasts with the protease inhibitors 1 mM phenylmethylsulfonyl fluoride and 2 mg of aprotinin and leupeptin each per ml. Binding reactions were as described previously (10). All competitor oligonucletides were added at 50-fold excess over test probe. Arrows 1 and 2 indicate shifts representing putative CRE binding proteins. These bands were present only with the *f*1 and *f*3 probes; they were subject to self competition but not to competition with the *f*2 oligonucleotide. Arrow 3 shows the migration of unshifted probe.

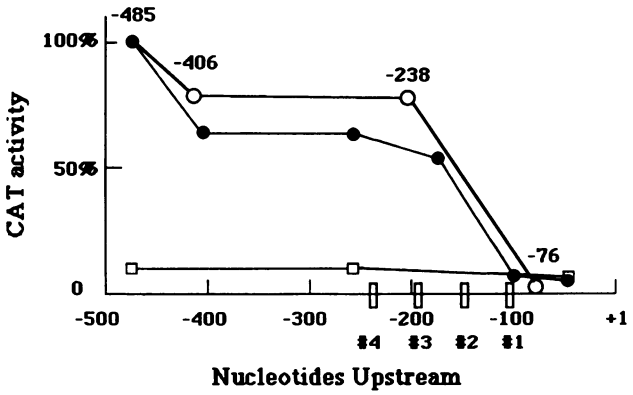

FIG. 4. Expression of deleted HCA and HCA$_{f1}$ promoters in C2 and HeLa cells. Deletions from −485 with respect to the transcription start site in the wild-type HCA promoter have been described previously (16). Deletions in the HCA$_{f1}$ promoter were made by digestion with *Nar*I, *Alu*I, and *Apa*I at positions −406, −238, and −76, respectively. The curves show CAT expression for each deletion series expressed as a percentage of maximum for each promoter. CAT assays were quantitated by scintillation counting of excised acetylated chloramphenicol spots from thin-layer chromatography plates. Positions of the CArG elements are indicated by the rectangles on the *x*-axis numbered 1 through 4. CAT expression is shown for wild-type HCA promoter in C2 (●) and HeLa (□) cells and for HCA$_{f1}$ promoter in HeLa cells (○).

in HeLa cells are similar. It appears that upstream elements in the modified HCA$_f$ promoter, in particular the CArG elements, maintain their transcriptional activating properties but are no longer tissue specific.

The apparent lack of an intrinsic muscle specificity of upstream transcription regulatory elements in the HCA promoter is consistent with our previous observation that the proteins which bind to these elements are present in all of the muscle and nonmuscle cells we have examined (10, 11). How then is tissue specificity determined? One possible explanation of our results is that the CRE and associated binding proteins displace a repressor from the −50 region of the HCA promoter. Indeed, the region −40 to −70 may be important since it contains an element with homology to the *myoD* consensus binding site (V. Sartorelli, K. Webster, and L. Kedes, unpublished data). To test for the possible disruption of a repressor binding site in the −50 region, we inserted the *f*1 oligonucleotide at position −86 relative to the transcription start site. This left the putative *myoD* binding site and flanking sequence intact. In this case, tissue specificity was again eliminated; high-level expression of the promoter with *f*1 inserted at −86 was again observed in HeLa cells as well as in C2 cells. By using β-actin expression as the standard, it was found that the −485 HCA construct containing *f*1 at position −86 expressed at a level of 114% ± 17% (n = 4) in HeLa cells compared with C2 cells. These experiments tend to refute a direct repressor displacement effect, although the mechanism of transcriptional activation by the CRE element in nonmuscle cells clearly may still involve overriding a repressor. An alternative possibility is that an as-yet-unidentified muscle-specific protein(s) interacts with the upstream ubiquitous DNA binding proteins and thereby activates the transcription complex. In this event, this protein(s) could be replaced in the modified HCA$_f$ promoter by a protein(s) which binds the c-*fos* CRE. The CRE binding protein(s) is found in most cells.

We have previously shown that cotransfection of the adenovirus type 5 E1A genes with HCA-promoter CAT constructs effectively eliminated expression of this promoter in muscle cells but had no effect upon nonmuscle-specific genes (28). Although it is not clear at what level this E1A-mediated transcriptional repression occurs, the effect was quite specific for muscle promoters. It is possible, therefore, that repression by E1A involves suppression of the postulated muscle-specific auxiliary transcription factor(s). If this is the case, then the E1A proteins should not mediate repression of HCA$_{f1}$ expression in nonmuscle cells. To test this possibility, an expression plasmid containing the adenovirus type 5 E1A region, pSVE1A (24), was cotransfected with HCA and HCA$_{f1}$ into C2 and HeLa cells (Fig. 5). Although pSVE1A effectively eliminated CAT expression from the normal HCA promoter in C2 cells, it had no effect upon expression directed by HCA$_{f1}$ in HeLa cells and only partially repressed HCA$_{f1}$-directed CAT expression in C2 cells. Although there are clearly other possible explanations, these data can be explained as follows. Expression of the HCA promoter in C2 cells requires the presence of one or more muscle-specific factors, and a step in the production, binding, or activation by this factor is sensitive to the E1A gene products. Expression of the HCA$_{f1}$ promoter in HeLa cells is directed exclusively through the c-*fos* CRE binding protein. This non-tissue-specific transcription pathway is not sensitive to repression by the E1A gene products. In fact, E1A proteins have been reported to *trans*-activate CREs (21). Expression of the HCA$_{f1}$ promoter in C2 cells, however, can occur in part by the non-tissue-specific, E1A-insensitive CRE route and in part via the normal tissue-specific mode requiring the muscle-specific factor that is sensitive to E1A-mediated repression. Hence, the expres-

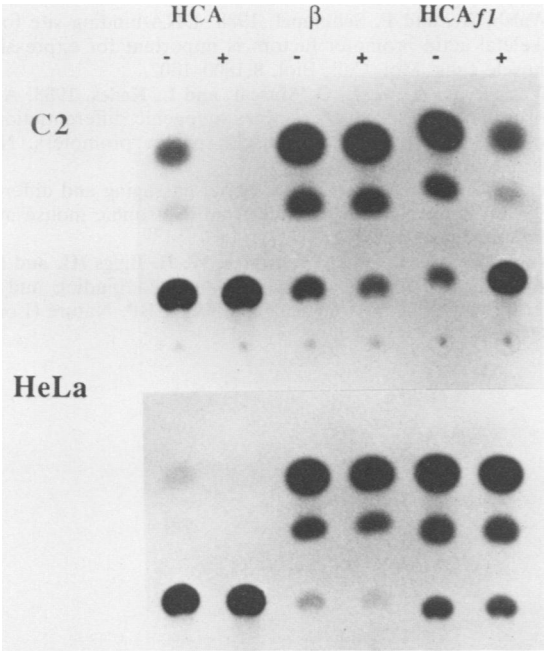

FIG. 5. Effect of pSVE1A cotransfection on expression of HCA and HCA_{f1} in C2 and HeLa cells. Assays were performed as described in the legend to Fig. 2. For lanes labeled +, 0.8 μg of the pSVE1A plasmid containing the adenovirus type 5 E1A genes (24) was cotransfected with 5 μg of the HCA promoter contructs. The total transfected DNA was 10 μg. Other conditions were as described in the legend to Fig. 2.

sion of HCA_{f1} in C2 cells is partially repressed by pSVE1A cotransfection.

To conclude, the tissue-specific expression of the HCA promoter can be overridden by the insertion of a c-*fos* basal promoter element containing an intact 8-bp CRE. Upstream sequences retain their normal potential for transcriptional activation, but now they do so in both muscle and nonmuscle cells. These upstream elements and their binding proteins, therefore, cannot alone account for the normal tissue specificity of this promoter. Although we have been unable to distinguish between proteins which bind to the HCA promoter from muscle and nonmuscle cells, it is possible that subtle differences exist. However, as noted above, this does not appear to be the case for CBF1, which is responsible for modulating the major transcriptional induction of the HCA gene in muscle cells. It is possible that the c-*fos* CRE binding protein substitutes for a muscle-specific factor which is involved in communicating the transcriptional effects of upstream regulatory sequences to the transcription complex, possibly in a manner analogous to the *Drosophila* Adh box A element (6). It should be noted that at least two previous studies have used the c-*fos* basal promoter to analyze regulatory elements: Pierce et al. (20) showed that the enhancer element which binds nuclear factor NF-κB conveyed lymphoid-specific expression when inserted upstream of position −71 of the c-*fos* promoter, and Walsh (226) showed muscle-specific regulation by chicken skeletal α-actin CArG containing oligonucleotides cloned upstream of c-*fos* at position −56. This latter construct lacks the CRE.

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